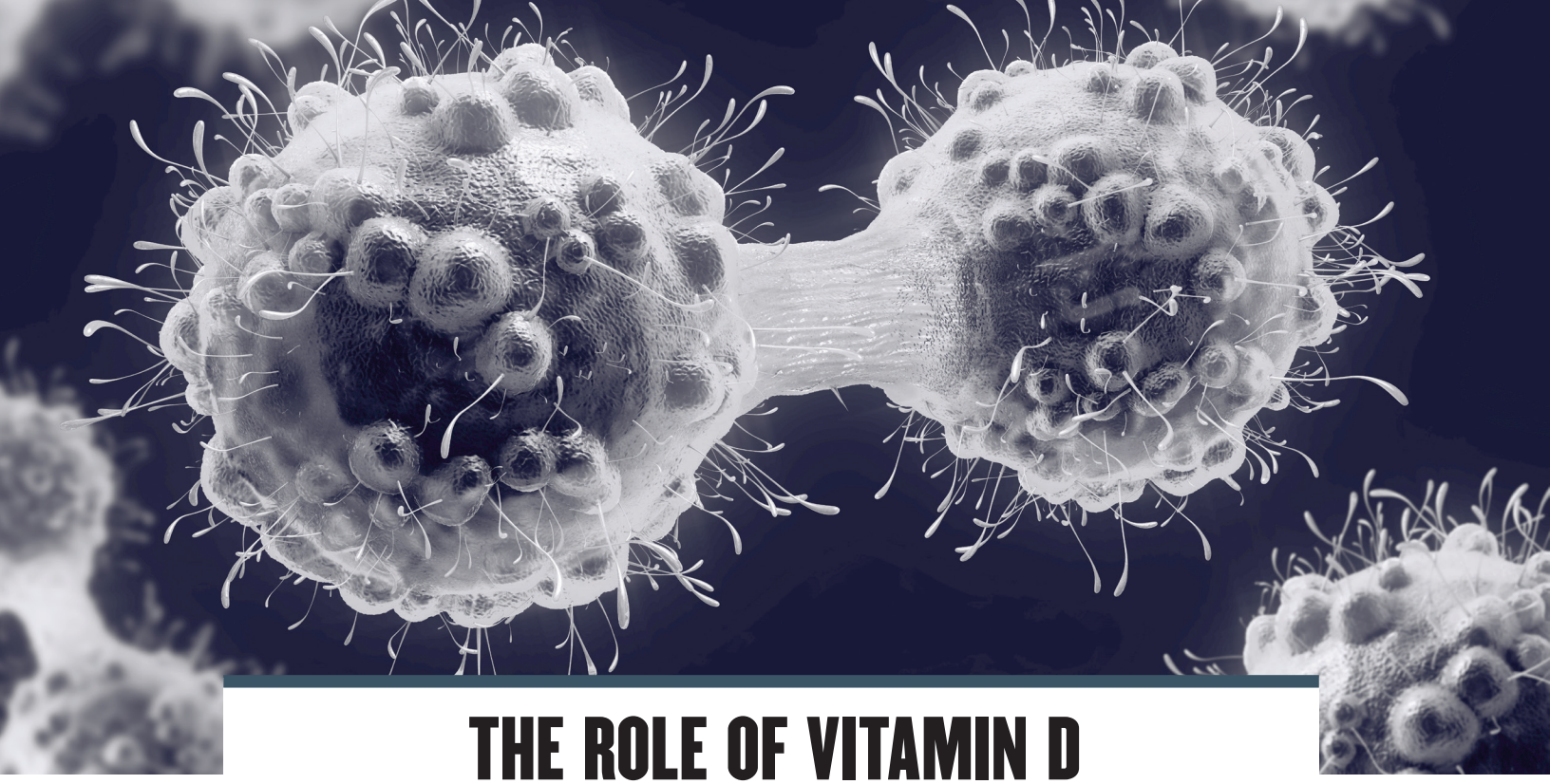
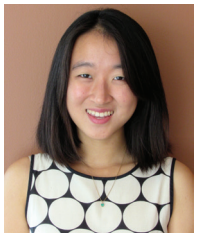


# THE ROLE OF VITAMIN D IN BREAST CANCER:

## Investigating Potential Inhibition Through Matrix Metalloproteinase 2


### Student Author



**Hyesoo Chae** is a professional student in the Purdue University College of Pharmacy. In the summer of 2015, she joined Dr. Dorothy Teegarden's laboratory through the Cancer Prevention Internship Program and has continued as a recipient of Purdue's Center for Cancer Research grant. She has worked as an intern at IU Health studying the clinical effect of vitamin K and warfarin on prothrombin time/international normalized ratio. Chae wishes to continue her studies in a clinical setting in both practice and research.

### Mentors

**Dorothy Teegarden** is a professor in the Department of Nutrition Science, and she serves as the associate dean for Research and Graduate Programs for the college of Health and Human Sciences. Teegarden received her BS from Antioch College, PhD from University of Chicago, and completed postdoctoral studies in both biochemistry and nutrition science at Purdue. The major focus of her research is the effect of vitamin D in preventing breast cancer progression, from very early stages to metastasis, in particular on regulation of energy and lipid metabolism. In addition, her laboratory investigates the mechanisms of how obesity impacts cancer development. Another research focus is the development of curricula to promote research skills in interdisciplinary research for both undergraduate and graduate students.

**Tomasz Wilmanski** obtained his BA degrees in psychology and philosophy from Tufts University, where he graduated with honors in 2011. After graduation, Wilmanski worked at the Block Medical Center for Integrative Cancer Treatment. During that time, he also obtained a post-baccalaureate certificate from Northwestern University. Wilmanski joined the Interdepartmental Nutrition Program at Purdue University in 2012, conducting research under the supervision of Dr. John Burgess and Dr. Dorothy Teegarden. Wilmanski graduated with a Doctor of Philosophy degree from Purdue University in August 2017.

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## Abstract

Breast cancer remains the second leading cause of cancer death among women in the United States, with 99% of breast cancer patients surviving five years after diagnosis if the tumor is localized. However, if the tumor has metastasized, the survival rate decreases to 22%. Epidemiological and animal studies, as well as previous studies from our laboratory in in vitro 3D cell culture models, support the hypothesis that vitamin D may inhibit breast cancer metastasis in humans, but the mechanisms remain unknown. The purpose of the present studies was to investigate the effect of the bioactive vitamin D metabolite, 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D$) on expression of matrix metalloproteinases (MMP) in MCF10CA1a breast epithelial cells. MMPs are a family of endopeptidases implicated in both cancer cell invasion and migration. First, the pattern of MMP expression in MCF10CA1a cells was investigated, focusing on MMPs 1,2,3,9 and 13. MCF10CA1a cells expressed only MMP2 mRNA at a detectable level. Treatment with $1,25(OH)_2D$ (10 nM,) reduced the expression of MMP2 by 32%±10 and 35%±7 at 2 and 5 days, respectively. Furthermore, the effect of $1,25(OH)_2D$ on migratory capability of MCF10CA1a cells was assessed using a wound healing assay. Pretreatment with $1,25(OH)_2D$ for 48 hours decreased cell migration by 44%±9.6 relative to vehicle. Overall, these studies support a potential role of MMP2 and migration in $1,25(OH)_2D$ prevention of breast cancer metastasis.

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## Keywords

breast cancer, metastasis, cell biology, matrix metalloproteinases

## INTRODUCTION

Breast cancer remains the second leading cause of cancer death and is the most frequently diagnosed cancer among women, with an expected 40,610 deaths due to breast cancer and 252,710 new cases to be diagnosed in 2017 (American Cancer Society, 2017). The survival rate of breast cancer patients is high, with 99% of patients surviving if the tumor is localized. However, if the tumor has metastasized, the survival rate decreases to 22% depending on the diagnosed stage (American Cancer Society, 2015; Yücel, et al., 2014). In addition, studies also show 70% of patients who are diagnosed with metastatic breast cancer at an advanced stage develop bone metastasis (Ahn et.al., 2013).

Cancer cells must break down physical barriers, including the extracellular matrix and basement membrane, in order for cancer cells to escape the primary tumor to a secondary site (thus metastasizing), which requires the expression of matrix metalloproteinases (MMPs). MMPs are zinc-dependent extracellular matrix (ECM) remodeling endopeptidases, which have the ability to degrade most components of the ECM. There are 26 known MMPs that are highly homologous yet differ in their substrate specificities. Each enzyme has a signal peptide to direct trafficking through the secretory pathway and a well-conserved, compact, catalytic domain (Radisky & Radisky, 2015). Studies show that MMPs are able to modulate the tumor microenvironment and regulate signaling pathways that control cell growth and migration, the immune system, and angiogenesis (Kessenbrock, 2010). Imbalances in the activity of MMPs can activate cellular processes that cause DNA damage and stimulate genomic instability (Wilmanski, Buhman, Donkin, Burgess, & Teegarden, 2017). MMPs may also directly induce phenotypic changes associated with the epithelial-mesenchymal transition, a developmental process that becomes activated during tumor progression (Wilmanski et al., 2017). MMPs could stimulate resistance to chemotherapeutics and drive tumor progression through proteolytic inactivation of the cell death receptor Fas and consequent inhibition of the intrinsic apoptosis pathway. Furthermore, findings suggest MMP overexpression may indicate a higher risk of poor prognosis in breast cancer (Ren et al., 2015).

Evidence suggests that vitamin D may protect against breast cancer as well as breast cancer metastasis. Vitamin D can be obtained through sun exposure, food, and supplements; however, vitamin D deficiency is prevalent in the United States The Teegarden laboratory has previously demonstrated

the effect of the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) on inhibiting metastasis of breast cancer cells in an in vitro model of the breast to bone metastasis (rMET) (Wilmanski et al., 2016). However, the molecular mechanisms for $1,25(OH)_2D$-mediated inhibition of metastasis are unknown. Therefore, further studies are needed to investigate the effect and mechanisms through which vitamin D inhibits breast cancer metastasis.

MMP activity is important to the first steps of metastasis to evade the primary site; however, the role of MMPs in $1,25(OH)_2D$-mediated inhibition of metastasis is unknown. Thus, the focus of this project is to investigate the role of $1,25(OH)_2D$ in altering the mRNA expression of MMPs to inhibit the invasion and migration of breast cancer cells.

## METHODS

### Cell Culture

In this study, MCF10CA1a cells were utilized. These cells are mammary epithelial cells that represent the metastatic stage of cancer progression with the potential to invade distant secondary organs. MCF-10CA1a cells were given as a gift from Dr. Julia Kirshner (Purdue University). For these experiments, cells were cultured in DMEM/F12, 1:1 supplemented with 5% horse serum, 100-units/ml Penicillin, and 100-ug/mL streptomycin. Cells were treated with vehicle (ethanol) or $1,25(OH)_2D$ (10 nm) (BIOMOL International) and delivered in 100% ethanol with a final ethanol concentration of <1%.The media was changed every 24 hours for the treatment period (Wilmanski et al., 2017).

### Scratch Wound Healing Assay

MCF10CA1a cells were plated in 6 well plates with 90% confluence and scratched with a 200 ul pipette tip. After 48 hours, the MCF10CA1a cells were treated with vehicle or $1,25(OH)_2D$ (10 nM). The new media containing 0.5% serum was added containing vehicle of $1,25(OH)_2D$, and images of wound closure were taken at time 0 and 24 hours. The results were quantified through TScratch.

### Real-Time Quantitative PCR

RNA was isolated with TriReagent. Reverse transcription of total RNA was performed using MMLV reverse transcriptase, and real-time quantitative PCR was performed using the Brilliant II SYBR Green QPCR Master Mix. The mRNA expression was normalized to 18S expression (Wilmanski et al., 2017). The primers used are shown in Table 1.

| **MMP1** | |
|---|---|
| Forward Primer | AGCGTGTGACAGTAAGCTAAC |
| Reverse Primer | TCCTCAGAAAGAGCAGCATCG |
| **MMP2** | |
| Forward Primer | AGATGCCTGGAATGCCAT |
| Reverse Primer | GGTTCTCCAGCTTCAGGTAAT |
| **MMP3** | |
| Forward Primer | GACAAAGGATACAACAGGGACCA |
| Reverse Primer | ACCGAGTCAGGTCTGTGAGT |
| **MMP9** | |
| Forward Primer | CAACATCACCTATTGGATCC |
| Reverse Primer | CGGGTGTAGAGTCTCTCGCT |
| **MMP13** | |
| Forward Primer | TGTAAAACGACGGCCAGT |
| Reverse Primer | CAGGAAACAGCTATGACC |

**Table 1.** Primer sequences for MMPs.

## RESULTS

MMPs have been implicated in the processing of cytokines, growth factors, and cell adhesion molecules, demonstrating their importance in sustaining a cancer cell's migratory potential. There is also evidence that supports MMPs having a role in tumor progression and metastasis, in shaping the tumor microenvironment, and in driving cancer progression and metastasis (Radisky & Radisky, 2015).

In order to study the impact of the active form of vitamin D, $1,25(OH)_2D$, on breast to bone metastasis, a unique 3-D cell model that recapitulates the transition steps that occur during breast cancer cell metastasis to the bone was utilized (Parikh, Belch, Pilarski, & Kirshner, 2014; Parikh, Minser, Rank, Glackin, & Kirshner, 2014). In this model system, the metastatic cells are plated in matrigel in an upper transwell, and the transwell is inserted into a well, which contains on the bottom a coating that is similar to a bone environment. The cells were treated only in the upper well with $1,25(OH)_2D$, and the number of viable cells that implant and grow in the bone matrix was assessed. Using this model, experiments in our laboratory showed that the number of viable metastatic MCF10CA1a breast cells that successfully metastasized to the bone matrix is drastically decreased by 70% with $1,25(OH)_2D$ treatment (Wilmanski et al., 2016). In addition, similar results were obtained with the metastatic MDA-MB-231 breast cancer cell

line, with a 20% reduction in cell viability in the lower bone matrix. It is noteworthy to emphasize that there was no 1,25(OH)$_2$D in the bone cell conditioned media, and thus, the altered phenotype and survival are due to the treatment in the upper well prior to movement into the bottom well. Therefore, the effects on metastasis to the bone are due, at least in part, to the treatment of the cells prior to and during invasion and migration from the upper well.

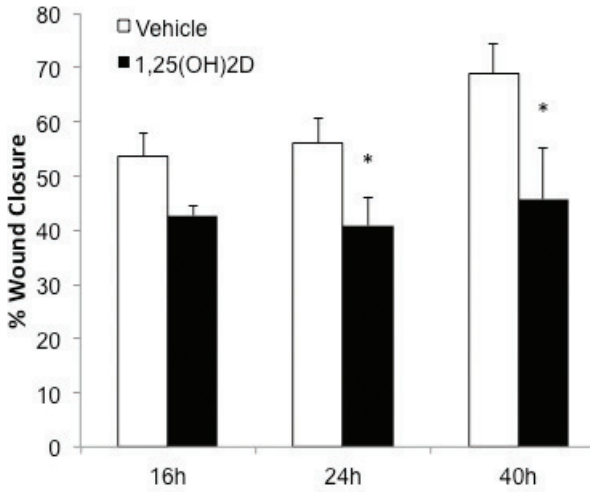


**Figure 1.** 1,25(OH)$_2$D inhibits migration of MCF10CA1a cells. MCF10CA1a cells were treated with vehicle or 1,25(OH)$_2$D (10nM) for 48 hours, after which each well was scratched with a 200ul pipette tip. New media containing 0.5% serum was added containing vehicle or 1,25(OH)$_2$D, and wound closure was monitored for 40 hours. *Indicates p-value < 0.05 relative to vehicle.

The

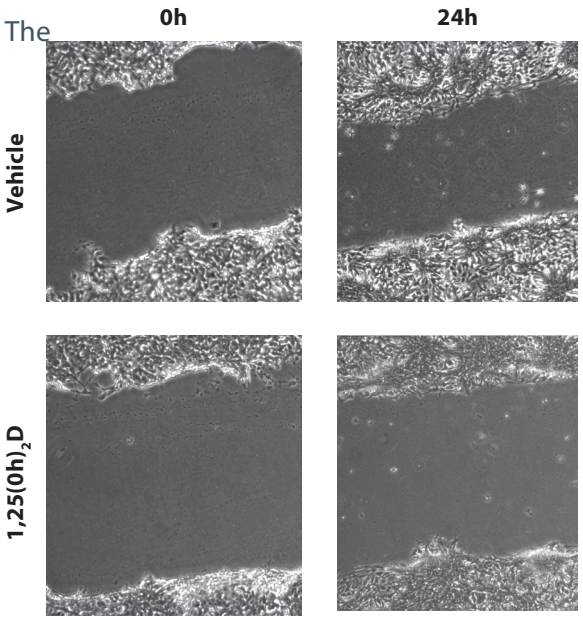


**Figure 2.** Representative images of MCF10CA1a breast epithelial cells from wound healing assay.

## Impact of 1,25(OH)$_2$D on Migration of MCF10CA1a

The role of 1,25(OH)$_2$D in the migration or movement of metastatic cells (MCF10CA1a cells) is important to understand in order to determine how metastasis is inhibited. Therefore, due to the important role of migration in metastasis, the effect of 1,25(OH)$_2$D on migration of MCF10CA1a cells was examined by a wound healing assay. As shown in Figure 1, pretreatment of cells with 1,25(OH)$_2$D for 48 hours decreased cell migration by 44%±9.6 relative to vehicle. The respective images can be seen in Figure 2.

## Impact of 1,25(OH)$_2$D on MMP mRNA Expression

The impact of 1,25(OH)$_2$D treatment on the mRNA abundance of a variety of MMPs was investigated through real-time quantitative PCR. MMP1, MMP3, MMP9, and MMP13 mRNA levels were undetectable in MCF10CA1a cells. However, MMP2 mRNA expression levels were detectable. MMP2 mRNA abundance was significantly downregulated after 48 hours and 5 days of 1,25(OH)$_2$D treatment. Treatment with 1,25(OH)$_2$D (10 nM) reduced the expression of MMP2 by 32% ± 10 and 35% ± 7 at 2 and 5 days, respectively, as seen in Figure 3.

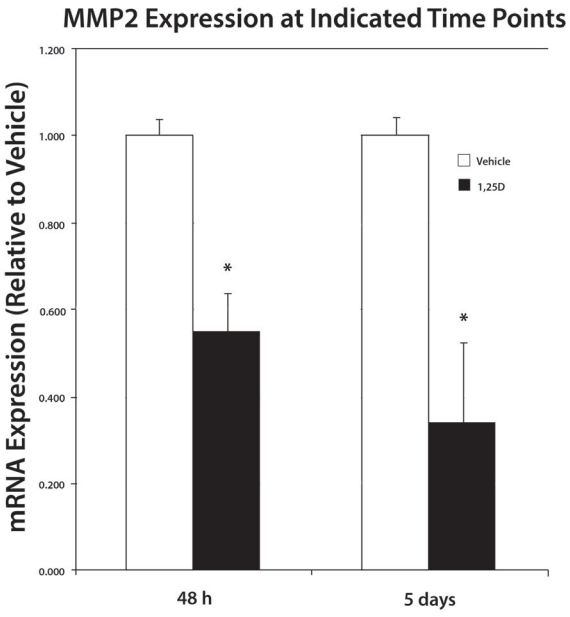


**Figure 3.** 1,25(OH)$_2$D decreases mRNA expression of MMP2 in MCF10CA1a cells. MCF10CA1a cells were treated with vehicle or 1,25(OH)$_2$D (10nM) for 48 hours and 5 days, after which mRNA was isolated and expression was quantified using qRT-PCR. *Indicates p-value < 0.05 relative to vehicle.

## DISCUSSION

Epidemiological and animal studies support the hypothesis that vitamin D may inhibit breast cancer metastasis in humans, but the mechanism remains unknown. There are a number of studies in humans that show that vitamin D status is associated with reduced risk for metastasis including breast cancer (Ren et al., 2012; Hauser, Walsh, Shrotriya, & Karafa, 2014; Neuhouser et al., 2008; Mohr, Gorham, Kim, Hofflich, & Garland, 2014). Further, perfusion with the circulating metabolite of vitamin D, 25-hydroxyvitamin D (25(OH)D), in an MMTV-PyMT mouse model of metastatic breast cancer increased levels of the bioactive vitamin D metabolite, $1,25(OH)_2D$ in the tumor, showing that increasing vitamin D status increases the local level of the active form of the vitamin in the tissue, In addition, tumor appearance was delayed, and lung metastasis decreased in this study (Rossdeutscher et al., 2014). In addition to studies in vivo, studies in cell models also support the hypothesis that $1,25(OH)_2D$ or its analogues inhibit metastasis, including invasion through matrigel and migration of MCF-7 and MCF10DCIS cells, and changes in expression of markers of metastatic capability. Therefore, evidence exist that $1,25(OH)_2D$ inhibits invasive and migratory capability of breast cancer cells in vitro and the metastasis of breast cancer cells in vivo. Despite illustrating this beneficial effect, the mechanistic basis for the inhibitory action of vitamin D on breast cancer metastasis remains poorly understood. It is critical to understand this mechanism to appropriately design clinical trials with well-defined and measurable outcomes, and to determine when and to whom interventions should be targeted.

Previous studies have shown the matrix metalloproteinase family to have a role in cancer and tumor metastasis. Of the 26 MMPs, MMP1, 2, 7, 9, 11, 13, and 14, along with the tissue inhibitors of MMPs, were quantified in invasive ducal breast tumors. MMP2 and MMP9 also have been examined as prognostic biomarkers in breast cancer (Radisky & Radisky, 2015). Thus, one mechanism by which $1,25(OH)_2D$ may inhibit metastasis is through downregulation of the expression of MMPs; however, this has not been examined.

The results of the current study demonstrate that $1,25(OH)_2D$ can downregulate the mRNA levels of MMP2, which may lead to a potential change in the protein expression. Therefore, vitamin D through its active form, $1,25(OH)_2D$, may be an effective agent in inhibiting invasion of breast cancer cells through downregulating MMP2 expression. In addition, the downregulation of MMP2 may play a role in inhibiting the migration of metastatic breast cancer cells.

Vitamin D is a food component and is synthesized by human bodies. Thus, it is a safe component to recommend for preventing metastasis. If the mechanisms of how vitamin D inhibits metastasis are determined, strategies can be developed to recommend specific intakes of the vitamin and may be targeted to specific populations to reduce the development of metastasis. Since breast cancer metastasis greatly reduces the survivability of the cancer, the impact of these studies, when translated to the clinic and general population, may have a significant impact to reduce mortality associated with breast cancer.

## FURTHER STUDIES

Our preliminary studies have shown that $1,25(OH)_2D$ inhibits the migratory capability of MCF10CA1a cells using a wound healing assay. To further explore the impact of $1,25(OH)_2D$, additional wound healing assays with other cell lines such as the MDA-MB-231 and 4T1 are necessary to ensure the effect is not specific to one cell line.

In addition, it is important to determine if the downregulation of MMP2 by $1,25(OH)_2D$ treatment plays a role in the inhibition of migration. To test this, inhibitors or activators of MMP2 activity can be used in the presence of vehicle or $1,25(OH)_2D$ and migration assessed. In particular, MMP2 targeting siRNA to knockdown MMP2 expression or overexpression of MMP2 in metastatic breast epithelial cells can be utilized. The MMP2 knockdown is expected to significantly inhibit both migration and invasion of metastatic breast epithelial cells, with $1,25(OH)_2D$ having no further effect on cell invasion. Similarly, we expect that a constitutive increase in MMP2 activity will significantly reduce or eliminate the $1,25(OH)_2D$ inhibition of migration of metastatic breast epithelial cells.

Because of the important role of MMP2 in invasion, the impact of $1,25(OH)_2D$ on invasive capability in the same breast epithelial metastatic cells (MCF10CA1a, MDA MB 231, and 4T1) also can be investigated. The invasion assay utilizes FluoroBlok matrigel coated transwell inserts with 8μm pore membrane. This cell culture system allows for quick invasive cell quantification using Calcein AM and subsequent fluorescence quantification. Fluorescently labeled cells present in the top chamber of the insert are shielded from bottom-reading fluorescence plate readers and microscopes by the FluoroBlok membrane.

## ACKNOWLEDGMENTS

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